(12)   EUROPEAN PATENT APPLICATION

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(54)   **Method for fluorescence correlation spectroscopy and device for carrying out the method**

(57)   A method for fluorescence correlation spectroscopy and a device for carrying out the method allow the determination of flow rates, diffusion coefficients, and volumetric concentrations.

Excitation light emitted from a light source is fed via a first optical fiber to a fiber coupler, and from there via a second optical fiber to a specimen. The fluorescent light emitted from specimen particles is fed via the second optical fiber to the fiber coupler, and from there via a third optical fiber to a detector.

Figure 1(A)

[see source for figure]

Description

The present invention relates to a method and a device for fluorescence correlation spectroscopy (FCS). The invention relates in particular to the determination of the flow rate and the thermal diffusion constant of particles that are self-fluorescent or that are labeled with fluorescent substances.

FCS is a known method for determining flow rates, diffusion coefficients, and volumetric concentrations. Such a method is described in D. Magde, E.L. Elson, W.W. Webb, Phys. Rev. Lett. 29 (1972), 705–708 and N.L. Thomson, "Topics in Fluorescence Spectroscopy, Vol. 1" (J.R. Lakowicz, Ed.), Plenum Press, New York 1991, 337–410.

In FCS, in a portion of the sample volume known as the observation volume, specimen particles are stimulated to produce fluorescent emissions by supplied excitation light. The fluorescent light is absorbed and is fed to a detector. The photon rate recorded at the detector fluctuates according to the number of particles present at the particular time in the observation volume. The various variables referenced above may be calculated from these signal fluctuations by using an autocorrelation function.

The necessary fluctuations appear only when the observation volume is so small that entering and exiting particles cause a change in the signal rate. Otherwise, in the static medium the number of particles entering and exiting the observation volume is always exactly the same, with the result that the signal rate remains essentially constant. In that case, it is not possible to evaluate the measured signal.

Typical observation volumes are in the range of less than one cubic micrometer. Achieving such observation volumes represents one of the major problems in FCS.

This problem can be solved by the use of a confocal microscope lens. In this regard, pinhole apertures are provided in the intermediate image plane in both the illumination beam path and the observation beam path of a standard microscope assembly, so that the image of the illumination aperture in the examined object falls exactly on the opening of the observation aperture. In this manner, the observed volume is limited to a very narrow region in the object plane.

Such a system has significant disadvantages resulting from the use of a lens. The lens is costly, large in size, must be adjusted in a complex procedure, and is sensitive to contaminants and vibration. Mobile use of such a unit is practically out of the question. The unit cannot be used for investigating locations that are difficult to access, such as conduits or inside the human body, since the specimen to be examined must always be placed directly on the lens.

The object of the invention is to provide a method for fluorescence correlation spectroscopy that easily achieves the necessary small observation volume, may be used in a mobile manner, and allows measurement in locations that are difficult to access, and a device for carrying out the method.

This object is achieved according to the invention by the method and device defined in the Claims.

The invention thus provides a method and a devices for fluorescence correlation spectroscopy that is simple and that allows mobile use, even in locations that are difficult to access.

In the method according to the invention, excitation light is fed from a light source via a first optical fiber to a fiber coupler, and from there via a second optical fiber to a specimen. Fluorescent light emitted from specimen particles is fed via the second optical fiber to the fiber coupler, and from there via a third optical fiber to a detector.

In one variation of the method, excitation light emitted from a light source is fed via an optical fiber to a specimen. The fluorescent light emitted from specimen particles is fed via an additional optical fiber to a detector.

One preferred embodiment uses monomodal optical fibers for several or all light transmissions between the light source, specimen, detector, and, optionally the fiber coupler.

In one preferred embodiment, the end of the optical fiber is submerged directly in the specimen. A further preferred embodiment provides that the end of the optical fiber 4 is separated from the specimen by a transparent layer.

When two separate fibers are used for excitation and detection, one or both of the fibers may be submerged in the specimen, or may be separated from the specimen.

In a further advantageous embodiment, the specimen-side end of the optical fiber is beveled; i.e., the normal to the specimen-side end surface of the second optical fiber forms an angle of at least 1° relative to the fiber axis. Preferably, an angle is formed for which the reflected portion of the excitation light no longer returns to the core of the fiber. When separate optical fibers are used for excitation and detection, one or both optical fibers may be beveled.

In another preferred embodiment, the end of the second optical fiber is designed as an elongated tip and is provided with a vapor-deposited metallic layer. The same applies for one or both of the two fibers when separate fibers are used for excitation and detection.

In one preferred embodiment, a plate is provided at a distance d that is less than 0.1 mm from the specimen-side end of the second optical fiber. When separate optical fibers are used for excitation and detection, the distance d may refer to each of the two fibers.

In the device according to the invention for carrying out the fluorescence correlation spectroscopy, a first optical fiber connects a light source to a fiber coupler. A second optical fiber connects the fiber coupler to a specimen, and a third optical fiber connects the fiber coupler to a detector.

Another form of the device according to the invention for carrying out the fluorescence correlation spectroscopy comprises a light source that is connected via an optical fiber to a specimen, and a detector that is connected via an additional optical fiber to the specimen.

In one preferred embodiment, individual or multiple optical fibers for connecting the light source, specimen, detector, and, optionally, the fiber coupler are composed of monomodal optical fibers.

A further preferred embodiment has a second optical fiber that is designed to be directly submerged in the specimen. For separate optical fibers for excitation and detection, one or both optical fibers may be correspondingly designed.

In another advantageous embodiment, the second optical fiber is separated from the specimen by a transparent layer. For separate optical fibers for excitation and detection, one or both optical fibers may be separated from the specimen in this manner.

In another preferred embodiment, the second optical fiber has a beveled specimen-side end; i.e., the normal to the specimen-side end surface of the optical fiber forms an angle of at least 1° relative to the fiber axis. Preferably, an angle is formed for which the reflected portion of the excitation light no longer returns to the core of the fiber. When separate optical fibers are used for excitation and detection, one or both optical fibers may be beveled in the manner described.

In a further advantageous embodiment, the specimen-side end of the second optical fiber is designed as an elongated tip and is provided with a vapor-deposited metallic layer. For separate optical fibers for excitation and detection, one or both fiber ends may be elongated and vapor-deposited.

One preferred embodiment of the device has a plate at a distance d that is less than 0.1 mm from the specimen-side end of the second optical fiber. For separate excitation and detection, the distance d may refer to each of the two fiber ends.

The preferred embodiments illustrated in Figures 1 through 7 are described in detail below.

Figures 1(A) and 1(B) and Figure 2(A) show the principle of the system according to the invention;

Figures 2(B) and 2(C) show various preferred arrangements of the specimen-side fiber ends with respect to one another when the excitation and detection occur with separate optical fibers.

Figure 3 shows a fiber end that is separated from a specimen by a separating layer.

Figure 4 shows a straight and a beveled fiber end next to one another.

Figure 5 shows an optical fiber that is elongated and provided with a vapor-deposited metallic layer.

Figure 6 shows an optical fiber in which a plate is provided at a distance d for varying the observation volume.

Figure 7 shows the test setup according to the invention when multiple specimens are to be measured simultaneously in the multiplex method.

Figure 8 shows an autocorrelation function measured from the signal rate, and Figure 9 illustrates the fluorescence intensity as a function of the concentration of fluorescent particles in a graph for a measurement example.

The principle of the method according to the invention and the device according to the invention is shown in Figure 1(A). A light source 1, typically a laser or laser diode, emits excitation light which is fed to a first optical fiber 2. The excitation light is supplied via a fiber coupler 3 and a second optical fiber 4 to a specimen 6.

The particles in the specimen for which the flow rate, diffusion coefficient, or volumetric concentration are to be measured are fluorescent or are labeled with fluorescent particles. The fluorescent light which is stimulated by the supplied excitation light is intercepted by the second optical fiber 4 and is fed via the fiber coupler 3 to a detector 10. It is advantageous to connect a spectral filter 9 upstream from the detector to absorb the portion of the excitation light that returns due to reflection on the boundary surface of the fiber.

The required small observation volume is achieved by the fact that the excitation light passes from the end of the second optical fiber 4 directly into the specimen 6. Because of the small diameter of the fiber of only several μm, the excitation light strikes the fluorescent particles in the specimen approximately in a cylinder having a diameter of several μm. Because in addition the depth of penetration of the light in the specimen is small in the longitudinal direction of the fiber, observation volumes in the range of several cubic micrometers are obtained.

According to the present invention, it is not necessary to use a microscope lens. The simplicity and robustness of the test setup allows even mobile use of an FCS unit. Due to the extreme compactness of the optical fibers, FCS measurements can also be performed in locations that are constricted or difficult to access, such as pipelines, combustion chambers of engines, or inside the human body.

When a four-port fiber coupler is used, it is advantageous for detection for the excitation light to be fed via an additional optical fiber 5 into an index-

matching liquid 7, as illustrated in Figure 1(B).The additional optical fiber prevents light reflection from occurring at the end of the optical fiber 5, since the index of refraction of the optical fiber and of the index-matching liquid are equal.

A variation of the invention is shown in principle in Figure 2(A). In this case, the excitation light emitted from the light source 1 is supplied to the specimen 6, and the detected fluorescent light is returned to the detector 10, by two separate optical fibers 4 and 8. These may be tailored to the various wavelengths of excitation light and fluorescent light. A spectral filter 7 upstream from the detector is advantageous, since here as well excitation light can pass into the detection fiber. In order to obtain a defined observation volume, during the measurement the ends of both optical fibers must occupy a position relative to one another that allows the excitation light to supply a region of the specimen from which fluorescent light can be detected. Figures 2(B) and 2(C) show several possibilities for positioning the excitation fiber 13 and the detection fiber 15. The observation volume 11 results from overlapping of the exiting light cone 12 of the excitation fiber and the acceptance cone 14 of the detection fiber. The excitation light can thus be deflected, even by a reflecting surface 16.

In one preferred embodiment, the optical fibers are composed of monomodal optical fibers. These have a much smaller cross section compared to multimodal optical fibers, and thus allow further reduction of the observation volume.

In one preferred embodiment, the end of one or both optical fibers is submerged directly into the specimen. This allows any location in the specimen to be easily measured, even when the location is difficult to access.

In another embodiment, illustrated in Figure 3, an optical fiber is not submerged directly in the specimen 22, but instead is separated from the specimen by a transparent layer 23. In this regard, the separating layer may also be part of a blood vessel wall. In this manner, FCS measurements may be performed even without direct contact with the specimen. This is particularly advantageous if, for example, the specimen represents a chemically or physically harmful environment for the optical fiber.

A further advantageous embodiment of the invention is illustrated in Figure 4. The specimen-side end of an optical fiber, which is composed of the core 32 and the sheathing 31 or 33, is beveled; i.e., the longitudinal axis 36 of the fiber forms an angle ϕ relative to the normal 35 to the fiber end face 34 that is greater than 1°, preferably between 1° and 10°. The portion of the excitation light that is reflected on the boundary surface 34 no longer returns to the core 32 of the optical fiber when the end of the fiber is beveled. This diminishes the background signal that occurs when the fluorescent light is detected.

Figure 5 shows a further preferred embodiment of the invention. The end of an optical fiber, which is composed of the sheathing 41 and the core 42, is elongated under heat, and the tip is provided on the sides with a vapor-deposited metallic layer 43. The vapor deposition is performed in such a way that an opening 44 remains directly at the tip which may have a diameter of down to 20 nm. In this manner, the observation volume, compared to the use of monomodal optical fibers, can once again be greatly reduced.

Elongated and metal vapor-deposited fiber tips are known from scanning near-field optical microscopy (SNOM), as described, for example, by E. Betzig, J.K. Trautman, Science 257 (1992), 189, and E. Betzig, R.J. Chichester, Science 262 (1993), 1422. In SNOM, a fiber tip is guided over a specimen surface, and an image of the specimen surface is obtained from the location-dependent intensity of the reflected light.

In the preferred embodiment shown in Figure 6, a plate 52 is provided at a distance d that is less than 0.1 mm from the end of an optical fiber 51. The observation volume 53 may be reduced even further in size by changing the distance d.

The described method and devices may also be combined with one another so that multiple specimens can be measured simultaneously. Signals may thus be detected in parallel using multiple detectors, or in series in a multiplex method.

The simple and inexpensive fiber technology is particularly suitable for multiplex operation (Figure 7). The excitation light is fed from the optical fiber 4 via the multiplexer 4a and additional optical fibers 4b to the specimens 5. Excitation light is fed to the various specimens in rapid alternation, and fluorescent light is detected from the specimens. In another variant, multiple light sources are connected via a multiplexer to a specimen. In this manner, simultaneous measurements may be made with excitation light of various wavelengths, which is advantageous when, for example, there are several types of particles with different fluorescence responses in the same sample volume.

Measurement Example 1

As an example for the use of the present invention, a system according to Figure 1 was used to measure the flow rate and concentration of fluorescence-labeled latex particles in water.

A 0.2% by weight dispersion of polystyrene latex particles with diameters of 110 nm was stirred in a vessel. The latex particles were labeled with the fluorescent dye tetramethylrhodamine.

A beveled optical fiber ($\phi = 8°$) with a core diameter of m = 3 μm was dipped into the specimen solution. Fluorescent excitation was provided by an argon ion laser at a wavelength of 514 nm. Detection was performed via a 550-nm long pass filter with a photomultiplier.

The signal recorded at the detector was sent to an electronic hardware correlator. This correlator calculates the correlation function k(t) for a variable time T of typically 30 s from the temporal progression of the amplitude of the detector signal I(t) according to the equation

[see source for equation]

From this correlation function and the average detector signal I_m

[see source for equation]

the normed autocorrelation function g(t)

[see source for equation]

is calculated. The flow of the fluorescent particles results in a step in the normed autocorrelation function g(t) (Figure 8). The position of this step is characterized by the value $t_{1/2}$. The crosshatched circles indicate the typical progression of an autocorrelation function in a resting latex dispersion for short correlation times.

The value of $t_{1/2}$ is obtained from the plot of the normed autocorrelation function g(t) by determining the initial and end values of the step. The difference between these two values is the step height h. The time at which the height of the step has dropped by half is $t_{1/2}$.

The fluorescent particles which flow with a velocity v past the end face of an optical fiber having a core diameter m are situated in the exit light from the core for an average time t_m

[see source for equation]

Within time 0.5 t_m, the autocorrelation function g(t) decays to half its value, i.e.,

[see source for equation]

The sought flow rate is thus calculated from the measured variable $t_{1/2}$ to give

[see source for equation]

Figure 8 shows the measured normed autocorrelation function g(t). The $t_{1/2}$ value was determined to be $t_{1/2}$ = 190 μs according to the above-described method. From this value an average flow rate of the latex particles was calculated to be 6.2 mm/s. Varying the rotational speed of the stirred results in greater or lesser flow rates.

Figure 9 shows the measured fluorescence intensity as a function of the concentration of the latex particles. A linear relationship exists between particle concentration and the measured intensity over a broad range, so that, using Figure 9 as a

calibration curve for unknown specimens, the concentration of such latex particles may be determined. Both graphs in Figure 9 show that the measurement curve or calibration curve is linear over a broad concentration range.

Measurement Example 2

If the motion of the fluorescent particles is caused only by the effect of diffusion, the measured normed autocorrelation function takes the form

[see source for equation]

where τ is the average diffusion time required by the particles to traverse a path having the length of the core diameter m. This time τ relates to m via the diffusion coefficient D:

[see source for equation]

For spherical particles with diameter a, the diffusion coefficient is calculated directly from the particle size:

[see source for equation]

In the above formula, k is the Boltzmann constant, T is the temperature, and η is the viscosity of the surrounding medium.

Thus, if a curve g(t) is fitted to a measured autocorrelation function according to the above formula, the size a of the particle may be determined from the value obtained for τ:

[see source for equation]

If the apparent hydrodynamic size of a fluorescent particle is modified by agglomeration onto another particle, this coalescence is measurable via a change in the value of τ.

Claims

1. Method for fluorescence correlation spectroscopy, **characterized in that** excitation light emitted from a light source (1) is fed via a first optical fiber (2) to a fiber coupler (3), and from there via a second optical fiber (4) to a specimen (6), and fluorescent light emitted from specimen particles is fed via the second optical fiber to the fiber coupler (3), and from there via a third optical fiber (8) to a detector (10).

2. Method for fluorescence correlation spectroscopy, characterized in that excitation light emitted from a light source (1) is fed via a first optical fiber (4) to a specimen (6), and fluorescent light emitted from specimen particles is fed via an additional optical fiber (8) to a detector (10), and the specimen-side ends

of the optical fibers are oriented with respect to one another so that the emitted excitation light passes into a region of the specimen from which the fluorescent light is detected.

3. Method according to Claim 1 or 2, characterized in that one or more of the optical fibers are monomodal optical fibers.

4. Method according to Claims 1, 2, or 3, characterized in that the specimen-side end of one or both of the optical fiber(s) is submerged directly in the specimen.

5. Method according to one of the preceding claims, characterized in that the specimen-side end of one or both of the optical fiber(s) is (are) separated from the specimen by a transparent layer.

6. Method according to one of the preceding claims, characterized in that the normal to the specimen-side end surface of the optical fiber for one or both optical fiber(s) forms an angle of at least 1° relative to the fiber axis, preferably an angle for which the reflected portion of the excitation light no longer returns to the core of the fiber.

7. Method according to one of the preceding claims, characterized in that the specimen-side end of one or both of the optical fiber(s) is designed as an elongated tip and is provided with a vapor-deposited metallic layer.

8. Method according to one of the preceding claims, characterized in that a plate is provided at a distance d that is less than 0.1 mm from the specimen-side end of an optical fiber.

9. Method according to one of the preceding claims, characterized in that the specimen-side end of one or both of the optical fiber(s) is connected to the input of a multiplexer having multiple outputs that are connected via optical fibers to multiple specimens, or that multiple light sources are connected via a multiplexer to the optical fiber leading to the specimen.

10. Device for carrying out the fluorescence correlation spectroscopy according to one of Claims 1 through 9, comprising a light source (1) that is connected via a first optical fiber (2) to a fiber coupler (3), a second optical fiber (4) that is connected via the fiber coupler (3) to a specimen (6), and a detector (10) that is connected via a third optical fiber (8) to the fiber coupler (3).

11. Device for carrying out the fluorescence correlation spectroscopy according to one of Claims 1 through 9, comprising a light source (1) that is connected via an optical fiber (4) to a specimen, a detector (10) that is connected via an optical fiber (8) to the specimen (6), and having the specimen-side ends of the optical fibers oriented with respect to one another so that emitted excitation light passes into a region of the specimen from which fluorescent light is detected.

12. Device according to Claims 10 or 11, characterized in that one or more optical fibers are monomodal optical fibers.

13. Device according to Claims 10, 11, or 12, characterized in that the specimen-side end of one or both of the optical fiber(s) are designed to be submerged directly in the specimen.

14. Device according to one of Claims 10 through 13, characterized in that the specimen-side end of one or both of the optical fiber(s) are separated from the specimen by a transparent layer.

15. Device according to one of Claims 10 through 14, characterized in that the normal to the specimen-side end surface of the optical fiber for one or both optical fiber(s) forms an angle of at least 1° relative to the fiber axis, preferably an angle for which the reflected portion of the excitation light no longer returns to the core of the fiber.

16. Device according to one of Claims 10 through 15, characterized in that the specimen-side end of one or both of the optical fiber(s) are designed as an elongated tip and is provided with a vapor-deposited metallic layer.

17. Device according to one of Claims 10 through 16, characterized in that a plate is provided at a distance d that is less than 0.1 mm from the specimen-side end of an optical fiber.

18. Device according to one of Claims 10 through 17, characterized in that the specimen-side end of one or both of the optical fiber(s) is connected to the input of a multiplexer having multiple outputs that are connected via optical fibers to multiple specimens, or that multiple light sources are connected via a multiplexer to the optical fiber leading to the specimen.

[see source for figures]

Figure 8

[Key]

strömende Flüssigkeit   = Flowing liquid

ruhende Flüssigkeit   = Resting liquid

Figure 9

[Key]

Fluoreszenzintensität   = Fluorescence intensity

Gew. %   = % by weight